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(54) Title: COMPOSITIONS AND METHODS FOR ENDOTHELIAL BINDING

(57) Abstract

Novel methods and compositions are provided for modulating homing of leukocytes, particularly lymphocytes, where the compounds are cross-reactive with or contain Neu5Ac2-3Galβ1-x[Fucα1-y]GlcNAc, where one of x and y is 3 and the other is 4. These compounds may be administered to a host associated with inflammation, to avoid the deleterious effects of leukocyte infiltration and for directing molecules to such sites. In addition, methods and compositions are disclosed for the inhibition of cancer metastases mediated by endothelial adhesion molecules. The present invention discloses that sialyl-Le^a and di-sialyl-Le^a, which are expressed at the surface of cancer cells, function as a binding partner for selectins, such as ELAM-1, which are expressed at the surface of endothelial cells. The present invention also discloses that selectins, such as ELAM-1, LECAM-1 and GMP-140, bind a carbohydrate domain common to both sialyl-Le^a and sialyl-Le^x. Antibodies, saccharides, glycoconjugates, enzymes, enzyme inhibitors and other molecules may be used in the methods of the present invention to inhibit the binding of malignant cells to endothelial cells for a variety of purposes *in vivo* and *in vitro*.

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Description

COMPOSITIONS AND METHODS FOR ENDOTHELIAL BINDING

Technical Field

The present invention is generally directed toward the modulation of leukocyte homing to provide therapies for inflammation and other pathogenic conditions associated with leukocyte infiltration into tissue; and toward the inhibition of cancer metastasis mediated by endothelial adhesion molecules, and more specifically, toward such inhibition through the use of saccharides, glycoconjugates, antibodies, enzyme inhibitors, and other agents, such as enzymes, which disrupt such binding of cancer cells to endothelia.

Background of the Invention

The bloodstream is the pathway for numerous cells which migrate throughout the body, monitoring conditions. Cells of the lymphoid and myelomonocytic lineages act to identify foreign substances, such as pathogens, aberrant cells, and some compounds, and remove them from the system. These cells have available a large variety of mechanisms for protecting the host from the foreign substance. Many of these mechanisms are highly destructive and result in cytotoxicity of native tissue, inflammation, degradation, and the like. Mechanisms may involve the production of superoxide, secretion of various degradative compounds, such as perforins, endocytosis, etc.

While in many situations these protective mechanisms are salutary, in many other situations, they are found to have detrimental effects, involving inflammatory lesions, such as myocarditis, inflammatory bowel disease, psoriasis, allergic contact dermatitis,

lichen planus, lymphoid hyperplasia in skin, inflamed synovia, reperfusion injury, etc.

In recent years, it has been shown that the migrating cells have specific surface membrane proteins associated with their homing or being directed to a particular site. Specialized venules including the high endothelial venules, serve as beacons for these cells, expressing proteins referred to as endothelial leukocyte adhesion molecules and addressins, which bind to the "homing receptors" or adhesion molecule surface membrane proteins of the migrating cells. After binding to the venules, the cells migrate by diapedesis, by mechanisms unknown, to the site of inflammation or injury.

Due to the difficulties in the current approaches in the treatment and prevention of diseases associated with or aggravated by the infiltration of migrating cells into an inflamed site, there is a need in the art for improved compositions and methods for inhibiting cell infiltration. The present invention fills this need, and further provides other related advantages.

One such related advantage pertains to cancer. Despite enormous investments of financial and human resources, cancer remains one of the major causes of death. Current cancer therapies cure only about fifty percent of the patients who develop a malignant tumor. In most human malignancies, metastasis is the major cause of death.

Metastasis is the formation of a secondary tumor colony at a distant site. It is a multistep process of which tumor invasion is an early event. Tumor cells locally invade host tissue barriers, such as the epithelial basement membrane, to reach the interstitial stroma, where they gain access to blood vessels ("hematogeneous metastasis") or lymphatic channels for further dissemination. After invading the endothelial layer of a vessel wall, the circulating tumor cells are dislodged into the circulation and arrest in the

precapillary venules of the target organ by adherence to endothelial cell lumenal surfaces, or exposed basement membranes. The tumor cells again invade the vascular wall to enter the organ parenchyma. Finally, the extravasated tumor cell grows in a tissue different from where it originated.

Most cancer cells fail to survive in the circulation and it appears that normally the lining of blood vessels acts as a barrier to tumor cell extravasation. Endothelial injury or perturbation increases tumor metastasis. In addition, certain factors, such as cytokines, have been shown to substantially increase the adhesion of cancer cells to treated endothelium in vitro. Interleukin 1 (IL-1) and tumor necrosis factor (TNF), which are cytokines, each stimulate the biosynthesis and expression of a cell surface receptor called ELAM-1 (endothelial leukocyte adhesion molecule). ELAM-1 is a member of a family of calcium-dependent cell adhesion receptors, known as selectins or selectins, which includes LECAM-1 and GMP-140 (also known as PADGEM or CD62). During an inflammatory response, ELAM-1 on endothelial cells functions as a "homing receptor" for leukocytes. Recently, ELAM-1 on endothelial cells was shown to mediate the increased adhesion of colon cancer cells to endothelium treated with cytokines (Rice and Bevilacqua, Science 246:1303-1306, 1989).

In most human malignancies, distant metastases are often too small to be detected at the time the primary tumor is treated. Furthermore, widespread initiation of metastatic colonies usually occurs before clinical symptoms of metastatic disease are evident. The size and age variation in metastases, their dispersed anatomical location, and their heterogeneous composition are all factors that hinder surgical removal and limit the concentration of anticancer drugs that can be delivered to the metastatic colonies. It has been estimated, for example, that in 1991 there will be over 60,000 deaths and

over 150,000 new cases from just colorectal cancer in the U.S. alone.

Due to the difficulties in the current approaches to the treatment and prevention of metastases, there is a need in the art for improved compositions and methods for inhibiting metastasis mediated by endothelial adhesion molecules. The present invention fills this need, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides compositions for modulating the leukocyte binding involving selectins to endothelial cells as sites of leukocyte exit from the blood. The compositions are characterized by binding to the selectin ELAM-1 or other selectin, and are at least in part other than polypeptide and substantially free of the natural polypeptide associated with the homing receptor, e.g., the cutaneous lymphocyte-associated antigen or LECAM-1. These compositions find particular use in inhibiting the homing of leukocytes, particularly lymphocytes, to sites of inflammation.

In one aspect, the present invention provides a method for modulating the binding of leukocytes or platelets to endothelial cells, the method comprising: adding to a combination of cells comprising leukocytes and endothelial cells expressing selectins or carbohydrate ligands thereof, in an amount sufficient to modulate the binding of leukocytes to endothelial cells, a compound capable of being cross-reactive and/or competitive with sialyl-Le^x, sialyl-Le^a or the cutaneous lymphocyte-associated antigen in binding to a selectin, wherein the compound is other than sialyl-Le^x when the selectin is ELAM-1. In one embodiment, the compound comprises sialic acid and fucopyranose bonded to a group comprising a conformationally constrained chain of at least 2 atoms.

In another aspect, the present invention provides a compound other than sialyl-Le^x comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to ELAM-1, LECAM-1 or GMP-140 for use within a method for inhibiting the infiltration of leukocytes into an inflammation site of a host. In one embodiment, the present invention provides a compound comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin for use within a method for inhibiting the infiltration of lymphocytes into an inflammation site of a host.

In yet another aspect, the present invention provides a compound comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin for use within a method for inhibiting the binding of platelets to endothelial cells.

In a related aspect, the present invention provides novel compounds. In one embodiment, the compound comprises a compound other than a naturally occurring sialyl-Le^a or sialyl-Le^x antigen comprising: sialic acid and fucopyranose bonded to a group comprising a conformationally constrained chain. In another embodiment, the compound comprises a compound other than a naturally occurring sialyl-Le^a or sialyl-Le^x antigen comprising: Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin. In yet another embodiment, the compound comprises a compound other than a sialyl-Le^x or sialyl-Le^a antigen comprising: Neu5Acα2-3Galβ1-x[Fucα1-y]R, wherein one of x and y is 3 and the other 4, R is a linker such as a saccharide or derivative, including ringed compounds such as constrained ring compounds, the compound being capable of binding to a selectin.

In addition, the present invention provides compositions and methods for the inhibition of cancer metastasis mediated by endothelial adhesion molecules. In one aspect, the present invention provides methods for inhibiting, within a biological preparation, the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x to endothelial cells expressing a selectin such as ELAM-1; or the binding of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to endothelial cells expressing a selectin such as ELAM-1. In one embodiment, the method comprises incubating the biological preparation with at least one agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin, wherein said agent is other than sialyl-Le^x when said malignant cells express sialyl-Le^x. In another embodiment, the method comprises incubating the biological preparation with at least one agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1, wherein said agent is other than sialyl-Le^x when said malignant cells express sialyl-Le^x. In another embodiment, the method comprises incubating malignant cells expressing sialyl-Le^a or di-sialyl-Le^a with at least one enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by the malignant cells. In yet another embodiment, the method comprises incubating said malignant cells with at least one enzyme that alters sialyl-Le^a or di-sialyl-Le^x of said malignant cells such that said malignant cells are incapable of binding to a selectin.

The present invention, in another aspect, provides compositions for use in methods for inhibiting the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites in a warm-blooded animal. In one embodiment, the composition comprises an agent that inhibits the binding of malignant

cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin. In another embodiment involving hematogeneous metastasis, the composition comprises an agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1. In a related aspect, compositions are provided for use in methods for inhibiting the spread of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to secondary sites in a warm-blooded animal. In one embodiment, the composition comprises an enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by the malignant cells. In another embodiment, the composition comprises an enzyme that alters sialyl-Le^a or di-sialyl-Le^a of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a such that malignant cells are incapable of binding to a selectin.

In another aspect, methods are provided for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells. In one embodiment, the method comprises incubating a biological preparation, containing endothelial cells expressing a selectin, with at least one agent capable of reacting with both sialyl-Le^a and sialyl-Le^x. In another embodiment, the method comprises incubating a biological preparation, containing endothelial cells expressing ELAM-1, with at least one agent capable of reacting with both sialyl-Le^a and sialyl-Le^x.

In another related aspect, compositions are provided for use in methods for inhibiting the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites in a warm-blooded animal. In one embodiment, the composition comprises an agent capable of reacting with both sialyl-Le^a and sialyl-Le^x. In another embodiment involving hematogeneous metastasis,

the composition comprises an agent capable of reacting with both sialyl-Le^a and sialyl-Le^x.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a graphic depiction of models for sialyl-Le^a and sialyl-Le^x.

Figure 2 describes pictorially a cell binding assay used to assess binding of human ELAM-1 transfected mouse cells to neoglycoproteins.

Figure 3 graphically illustrates the relative binding of human ELAM-1 transfected mouse cells to certain neoglycoproteins.

Figure 4 graphically illustrates the relative binding of human ELAM-1 transfected mouse cells to certain neoglycoproteins.

Figure 5 graphically illustrates the inhibition of binding of human ELAM-1 transfected mouse cells to immobilized sialyl-Le^a-HSA (human serum albumin) by soluble sialyl-Le^a-HSA.

Figure 6 graphically illustrates the inhibition of binding of human ELAM-1 transfected mouse cells to immobilized sialyl-Le^a-PA (polyacrylamide) by soluble sialyl-Le^a-PA.

Figure 7 graphically illustrates the selective binding of LECAM-1 transfected cells to neoglycoproteins.

Figure 8 graphically illustrates the binding of lymphocytes to neoglycoproteins.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

ELAM-1 (also known as "E-selectin") is a vascular selectin. ELAM-1 recruits neutrophils during

acute inflammation, but during chronic inflammation is selectively found in skin, binding skin homing lymphocytes, i.e., it doubles as a skin vascular addressin.

GMP-140 (also known as "G-selectin") is a vascular selectin which binds neutrophils and monocytes early in inflammation; it is also expressed on stimulated platelets.

LECAM-1 (also known as "L-selectin") is a vascular selectin involved in neutrophil, monocyte, etc., extravasation in acute inflammation, and lymphocyte homing to peripheral lymph nodes and some sites of chronic inflammation.

Selectins (formerly referred to as "LEC-CAMs") are defined structurally, being a lectin with the same or similar structural motifs as LECAM-1 (the Mel-14 antigen).

Addressins are defined as any tissue specific vascular adhesion molecule involved in lymphocyte homing. The peripheral lymph node addressin (PLN) is a glycoprotein (carbohydrate ligand) for the lymph node homing receptor (selectin) LECAM-1. The mucosal addressin is a 60kD glycoprotein.

Antibody, as used herein, includes both monoclonal and polyclonal antibodies and may be an intact molecule, a fragment thereof, or a functional equivalent thereof. The antibody may be genetically engineered. Examples of antibody fragments include $F(ab')_2$, Fab', Fab and Fv.

Saccharide, as used herein, includes oligosaccharides, and may be naturally derived, synthetically prepared, portions of either, and derivatives of any of the foregoing.

Glycoconjugate, as used herein, includes a saccharide which is linked to a non-saccharide molecule, e.g., a lipid or a polypeptide.

As noted above, the present invention, in one aspect, provides for the prophylactic and therapeutic

modulation of homing of leukocytes, particularly lymphocytes, to sites of inflammation. The compositions are characterized by binding to an endothelial cell or leukocyte lectin adhesion molecules or if the selectin family are cross-reactive with at least one epitope of sialyl-Le^x, and sialyl-Le^a, are other than sialyl-Le^x and will usually involve at least about three saccharide monomer units.

The active structures of the compositions which find use will be under about 5,000 molecular weight and may be under about 3,000 molecular weight, generally being at least about 800 molecular weight. The compositions themselves may have multiple copies of the active structures, bonded to a common backbone (polymeric chain such as polyacrylamide, polyvinylalcohol, cyclodextrans, etc.), liposomes, and the like. Any compound which has the above-indicated characteristics of cross-reactivity in binding to at least one of ELAM-1, LECAM-1, or GMP-140, is other than sialyl-Le^x or protein conjugate thereof and is physiologically and pharmacokinetically acceptable may be employed. The compounds may be naturally occurring or synthetic and may be saccharides, synthetic organic compounds or the like.

Of particular interest are the sugars sialic acid (neuraminic acid), galactose, fucose, or derivatives thereof, combined to form an oligosaccharide derivative. The sugar monomers may be further derivatized by having up to four, usually not more than three groups bound to carbon, nitrogen or oxygen, which groups may include an additional sugar, such as sialic acid, glucosamine, galactose, glucose, fucose, etc., alkyl groups, such as methyl, ethyl, acyl groups, such as acetyl, etc., and the like. The site of substitution will not interfere with the binding of the compound to its complementary receptor or lectin domain but may provide such advantages as improved pharmacokinetics, stability, ease of synthesis, reduced toxicity, enhanced affinity, and the like.

Leukocytes which may be modulated as to their homing to tissues, where the leukocyte or endothelial cell is expressing the selectin, include neutrophils, T-lymphocytes and B-lymphocytes, platelets, etc. These cells are found to home to a variety of injured, diseased, or otherwise pathogenic states, particularly associated with inflammation. Infiltration of these cells can be associated with such conditions as psoriasis, allergic contact dermatitis, lichen planus, lymphoid hyperplasia in the skin, non-specific chronic dermatitis, pityriasis lichenoids et varioformis acuta, granuloma annular, cutaneous drug eruption, pityriasis rubra pilaris, inflamed synovia, reperfusion injury, or the like. Sites to which the leukocytes migrate include peripheral lymph nodes, skin, Peyers patches, spleen, mesenteric lymph nodes (mucosal tissue), synovium, and other lymphoid and extralymphoid tissues and sites of inflammation.

The subject compositions may be prepared in accordance with conventional ways or isolated from a natural source, e.g., milk. Descriptions of the preparations of sialyl-Le^x, sialyl-Le^a, the common portions of the two compounds, namely Neu5Ac2, 3Galβ-x[Fucα1-y]GlcNAc, wherein one of x and y is 3, and the other is 4, and cross-reactive derivatives thereof are illustrated by the synthesis of a variety of sugars which may be found in Paulsen, (1982) Angew. Chem. Int. Ed. 94:184; Fugedi et al., (1987) Glycoconjugate J. 4:97; and Okamoto and Goto, (1990) Tetrahedron 46:5835; Kameyama et al., (1991) Carbohydr. Res. 209:C1; and Palcic et al., (1989) Carbohydr. Res. 190:1-11.

The compounds of this invention will be other than the naturally occurring sialyl-Le^a or sialyl-Le^x antigens found as polysaccharide markers on human cells. The compounds are characterized by having a structure which comprises, or is immunologically cross-reactive with a structure that comprises, a fucopyranose and a sialic acid or derivative thereof in a spatial conformation

associated with both sialyl-Le^a and sialyl-Le^x. Thus, the two sugars, sialic acid and fucopyranose, will be bonded to a chain which permits the sugars to assume the proper orientation and spatial conformation, preferably provides restraint in maintaining such conformation.

The backbone chain may be from 10 to 20, usually 3 to 8, preferably 3 to 7, more preferably 5 to 6 atoms, which may be carbon, nitrogen or oxygen, and may involve alicyclic, cyclic, heterocyclic or aromatic units or combinations thereof. Where a sugar is at least a portion of the backbone, desirably the sialic acid group will be present as the non-reducing terminal sugar of a disaccharide, where the other sugar is preferably galactose, and the disaccharide is separated by from about 1 to 4, preferably 1 to 3, particularly 2 atoms, usually carbon and optionally oxygen atoms, from the fucopyranose. The group serving as the separating chain desirably will be conformationally constrained, particularly as a cyclic or heterocyclic group. The group may be substituted with one or more oxy (including hydroxy) groups. By conformationally constrained for cyclic groups are intended ranges of from 3 to 7, usually 5 to 6 annular members, or sterically hindered compounds, or other structured where the atoms of the chain are inhibited from free rotation. The sialyl and fucopyranose groups may be cis or trans, equatorial or polar, in their spatial positions, usually trans.

For the most part, the subject compositions have as their core structure:

Neu5Acα2-3Galβ1-x[Fucα1-y]R

wherein R is glucose or derivatives, e.g., glucosamine, N-acetyl glucosamine, etc., and other ring structures including constrained cyclic structures, where any of the positions of the core structure may be substituted without interfering with the binding to selectins. Sites for substitution include the available positions of galactose, glucose, and fucose, particularly

with a sugar, e.g., sialic acid, glucosamine, N-acetyl glucosamine, glucose, neuraminic acid, fucose, disaccharides thereof, etc., where the nitrogen atoms may be alkylated or acylated; and the like.

Of particular interest are compounds comprising a cyclic group to which fucose and a disaccharide with neuraminic acid as the non-reducing terminal sugar is bonded, where the fucose and disaccharide are separated by from 2 to 3, particularly carbon atoms and optimally an oxygen atom. Thus the cyclic compound may be of 5 to 7 annular members, particularly 6 annular members, and may include 1,2-cyclohexanediol, 1,3-cyclohexanediamine, 1,2-cyclohexanolamine, 1,2-cyclopentandiol, 2,3- or 3,4-dihydroxypyran, and the like. The positions may be cis or trans, preferably trans.

For a variety of purposes, the saccharidic compounds may be conjugated to other compounds, such as lipids, detergents, e.g., non-ionic detergents, such as polyaklyleneoxy groups, with alkylene of from 2-3 carbon atoms, usually under about 5kDal, naturally occurring or synthetic organic compounds where the active structure is under about 2kDal, which may be alicyclic, aromatic, acyclic or heterocyclic, polymeric compounds, such as physiologically acceptable polymers, e.g., acrylates, proteins, or the like which may be under about 100kD or more.

Proteins which may find use as carriers include serum albumin, casein, gelatin, etc. Conjugates may be prepared as immunogens to produce antisera or monoclonal antibodies specific for the binding epitope. Thus, antibodies could be used to inhibit homing of leukocytes, e.g., neutrophils, lymphocytes or other leukocytes. Anti-idiotypic antibodies may be prepared which would compete with the binding epitope for the selectins to prevent lymphocyte infiltration.

The subject compounds may be conjugated to the carriers directly, but more usually through a spacer.

Various spacers are known for linking to proteins, particularly spacers incorporating aromatic groups, e.g., phenylene, substituted with from 1 to 2 amino groups where the other functionality may be a carboxylic acid, aldehyde, mercaptan, activated olefin or the like. In bonding the spacer to the saccharide through an amino group, the linkage may provide for retention of the anomeric configuration of the reducing sugars or reductive amination may be employed resulting in an aminoalditol (Kallin et al., Glycoconjugate J. 3:311, 1986).

Based on the configuration of the binding epitope, using computer-assisted design, synthetic organic compounds can be devised which would compete with the binding epitope for the addressin.

The subject compositions may be administered in any convenient way, depending upon the particular nature of the composition. Various physiologically acceptable media may be employed, such as deionized water, saline, phosphate buffered saline, aqueous ethanol, and the like. Depending upon the nature of the compound, it may be administered typically, parenterally or orally, subcutaneously, intravascularly, topically, peritoneally, and the like. The particular dosage will vary with the frequency of administration, the manner of administration, the activity of the compound, the indication being treated, and the like.

As noted above, the present invention is also generally directed towards compositions and methods for the inhibition of cancer metastasis mediated by endothelial adhesion molecules. More specifically, the disclosure of the present invention shows that antibodies, saccharides, glycoconjugates therefrom, enzymes or enzyme inhibitors may be used to inhibit the binding of malignant cells to endothelial cells for a variety of purposes in vivo and in vitro.

As described above, metastasis is a multistep process. During metastasis, cancer cells circulate

through the microvascular and lymph systems and then migrate through the walls of the blood or lymph vessels to establish a new and aggressive tumor at a secondary organ site. A critical step in the metastasis process is the adherence of circulating cancer cells to the endothelial lining of blood vessel or lymph vessel walls. As disclosed within the present invention, the carbohydrates sialyl-Le^a and di-sialyl-Le^a, which are expressed at the surface of certain cancer cells, function as a ligand (i.e., binding partner) for selectins, such as ELAM-1, which are expressed at the surface of endothelial cells. Therefore, for those cancer cells, metastasis involves the adherence of cancer cells to the endothelial cells via the binding of sialyl-Le^a and/or di-sialyl-Le^a on the cancer cells to adhesion molecules on endothelial cells. Other cancer cells express predominantly sialyl-Le^x, or sialyl-Le^x and sialyl-Le^a (and/or di-sialyl-Le^a). The present invention discloses that selectins, such as ELAM-1, bind a carbohydrate domain common to both sialyl-Le^a and sialyl-Le^x on malignant cells, and therefore agents can be produced which are capable of binding to both. Other sialylated glycoconjugates may be expressed as well which possess the common domain.

Inhibition of the initial binding event between selectins and sialylated structures by the methods of the present invention prevents the adhesion of metastatic cells to the endothelial lining of blood or lymph vessel walls, thereby eliminating the spread of metastatic cells to secondary organs. Suitable blocking agents include those which inhibit the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a, or sialyl-Le^x (including or not di-sialyl-Le^x), to endothelial cells expressing selectin adhesion molecules such as ELAM-1. Representative agents include antibodies, saccharides and glycoconjugates therefrom, enzymes and enzyme inhibitors.

The antibodies employed in the present invention may be polyclonal or monoclonal antibodies. Briefly,

polyclonal antibodies may be produced by immunization of an animal and subsequent collection of its sera. Immunization is accomplished, for example, by a systemic administration, such as by subcutaneous, intrasplenic or intramuscular injection, into a rabbit, rat or mouse. It is generally preferred to follow the initial immunization with one or more booster immunizations prior to sera collection. Such methodology is well known and described in a number of references.

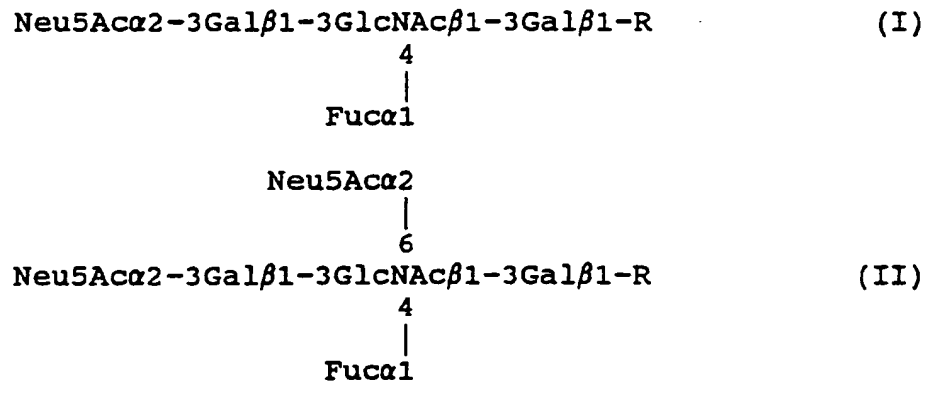
Monoclonal antibodies (MAbs) suitable within the present invention include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (i.e., antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods known by those skilled in the art. Human antibodies and chimeric human-mouse antibodies are advantageous because they are less likely than murine antibodies to cause the production of anti-antibodies when administered clinically.

MAbs may be generally produced by the method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol. 6:511-519, 1976). Briefly, the lymph nodes and/or spleens of an animal immunized with molecules containing sialyl-Le^a or di-sialyl-Le^a are fused with myeloma cells to form hybrid cell lines ("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin and, like the myeloma cells, has the potential for indefinite cell division. Hybridomas are selected for producing antibodies that bind the desired carbohydrate structure by screening appropriate neoglycoconjugates. An alternative to the production of MAbs via hybridomas is the creation of MAb expression libraries using bacteriophage and bacteria (e.g., Sastry et al., Proc. Natl. Acad. Sci USA 86:5728, 1989; Huse et al., Science 246:1275, 1989). Selection of antibodies exhibiting appropriate specificity may be performed in a

variety of ways which will be evident to those skilled in the art. Typically, such antibodies will selectively bind with an affinity of about 10^7 liters/mol or higher.

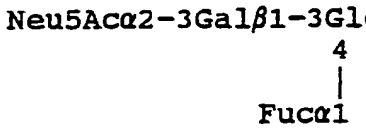
Representative examples of MAbs suitable within the present invention include N-19-9 and HECA-452 for sialyl-Le^a, and FH-7 for di-sialyl-Le^a. MAb N-19-9 is available from ATCC (American Type Tissue Collection, Rockville, Maryland) as ATCC HB 8059 or may be produced as described in U.S. Patent No. 4,471,057 (and Somatic Cell Genet. 5:957-971, 1979; J. Biol. Chem. 257:14365, 1982). MAb HECA-452 may be produced according to Duijvestijn et al., Am. J. Path. 130:147-155, 1988. FH-7 may be produced according to Nudelman et al., J. Biol. Chem. 261:5487, 1986.

In addition to antibodies which are capable of binding to sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, saccharides and glycoconjugates therefrom may also inhibit the binding of metastatic cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelia. As used herein, the terms "sialyl-Le^a" and "di-sialyl-Le^a" represent structures I and II, respectively, as follows:



Neu5Ac represents sialic acid; Gal represents galactose; GlcNAc represents N-acetyl-glucosamine; Fuc represents fucose and R is typically a ceramide (with a glucose residue interposed) or a protein. Sialyl-Le^x is an isomer of sialyl-Le^a wherein the Gal-GlcNAc linkage is β1-4 and

the Fuc-GlcNAc linkage is α1→3. Saccharides suitable within the present invention include the carbohydrate portion of sialyl-Le^a or di-sialyl-Le^a (i.e., formula I or II minus R), and derivatives of either, including those which cross-react with both sialyl-Le^a and sialyl-Le^x. Derivatives of these compounds include substitution of individual saccharide residues with other saccharide residues and/or with non-saccharide molecules such as hexyl rings without hydroxyl groups. For example, the internal GlcNAc may be replaced with another saccharide residue such as a glucose (Glc). Alternatively (or in addition to substitutions), the carbohydrate portion of sialyl-Le^a, di-sialyl-Le^a, or derivatives thereof, may be truncated by deletion of one or more saccharide residues. For example, a tetrasaccharide may be created with the structure:

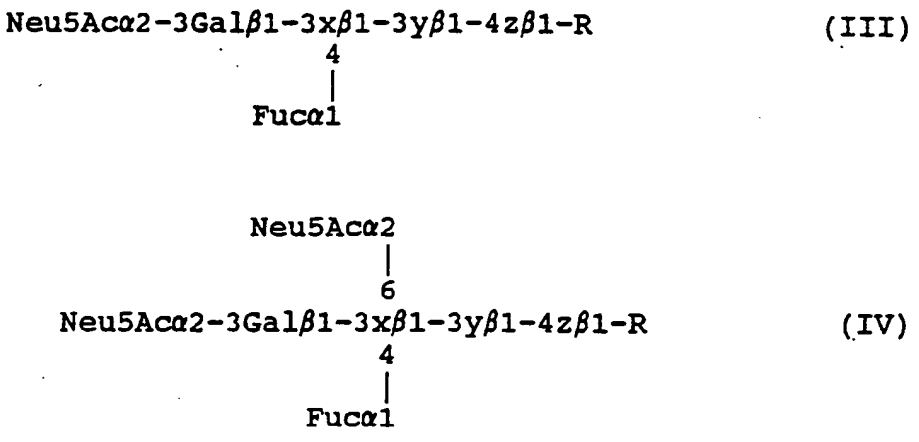


Given the teachings described herein, it will be evident to those skilled in the art that other saccharides will be suitable within the present invention.

A saccharide may be coupled to a non-saccharide molecule to form a glycoconjugate. For example, a saccharide may be linked to a polyacrylamide. Alternatively, a saccharide may be linked to a lipid. Typical lipids include ceramide, i.e., sphingolipid bases which are acylated on the amine with a fatty acid. For example, sialyl-Le^a, di-sialyl-Le^a, or a saccharide cross-reaction with sialyl-Le^a and sialyl-Le^x may be linked to a ceramide. Alternatively, a saccharide may be bonded to an amino acid or an amino acid-containing molecule, such as a peptide, a polypeptide or a protein. Saccharides are naturally linked to an amino acid or amino acid-containing molecule via the hydroxyl group of a serine or threonine

amino acid residue, but can also be linked through other groups such as an amino group.

Saccharides and glycoconjugates provided by the present invention may be represented by structures III and IV as follows:



R includes H, OH, lipid, ceramide, or one or more amino acids; x, y and z are independently selected from saccharides, or either y or z or both may be absent.

Numerous methods for preparing saccharides and glycoconjugates are well known to those skilled in the art. Saccharides may be prepared synthetically using chemical, and/or enzymatic, reagents and techniques. For example, sialyl-Le^a saccharides have been prepared by enzymatic synthesis (e.g., Palcic et al., Carbohydr. Res. 190:1-11, 1989). Glycoconjugates may be prepared, for example, through reductive amination. The method of Zopf et al. (Meth. Enzymol. 50:171-175, 1978; Jeffrey et al., Biochem. Biophys. Res. Commun. 62:608-613, 1975) involves 4-aminophenethylamine derivatives of saccharides via reductive amination using sodium borohydride. In brief, sugars are first reacted with the amino reagent by dissolving them in the neat reagent for 15 hours. Sodium borohydride in ethanol is then added. After 5 hours, the product is separated from the reagent by gel filtration and ion exchange chromatography. The derivatives may then be coupled to a molecule containing a group which is

reactive with amines. The same amine derivative may be coupled to saccharides using sodium cyanoborohydride. (Svensson et al., J. Immunol. Meth. 25:323-335, 1979). In brief, a sugar is dissolved in water, and the same volume of amine (a 170-fold molar excess) is added together with sodium cyanoborohydride (a ten-fold molar excess). The reduction is performed at pH 8 for 48 hours, and the product purified by gel chromatography. Coupling to different molecules, such as proteins, may be performed by the isothiocyanate coupling method.

Another example of a reagent suitable for preparing glycoconjugates by reductive amination is p-trifluoroacetamidoaniline (TFAN). The reductive amination reaction is carried out in aqueous solution overnight at pH 5-6 with sodium cyanoborohydride as the reducing agent. Typically, a 5-fold excess of TFAN is used. TFAN-derivatized saccharides are generally protected from oxidation by N-acetylation, e.g., by treatment with methanolic acetic anhydride, to yield TFAc-derivatives. Prior to conjugation, the N-trifluoroacetamido protective group is removed by treatment of the TFAc derivative with aqueous ammonia or 0.5 M sodium hydroxide for 3 hours. Conjugation of the derivatives to molecules, for example to proteins such as bovine serum albumin (BSA), may be achieved by isothiocyanate coupling methods. Other examples of suitable reagents and reactions include p-tetradecylaniline derivatives of saccharides and the preparation of aminoalditols by oxidation of saccharide TFAN derivates with cerium ammonium sulfate (Lindenberg et al., J. Reprod. Fert. 89:431-439, 1990).

Multivalent carbohydrate drug candidates can be prepared from N-acrylol glycosylamines which are produced by acylating glycosylamines with acryl chlorides. The N-acrylol glycosylamines are co-polymerized with acrylamide using a radical initiator in aqueous solution to produce multivalent carbohydrate polymers in which the degree of substitution is determined by the molar ratio of the

reactants (Kallin et al., J. Carbohydrate Chem. 8:597-611, 1989). Using this method, sialyl-Le^a was co-polymerized to form a multivalent carbohydrate polyacrylamide ("SLe^a-PA," e.g., Figure 6). This multivalent carbohydrate drug candidate is non-toxic and water soluble. The molecular weight and hapten density can be determined by altering the ratio of reactants and the reaction time.

The inhibition of the binding of cancer cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelia has a variety of in vitro and in vivo uses. Sialyl-Le^a and di-sialyl-Le^a are type 1 carbohydrate chains (i.e., have a Galβ1→3GlcNAc polylactosamine unit structure) and sialyl-Le^x is a type 2 carbohydrate chain (i.e., has a Galβ1→4 GlcNAc polylactosamine unit structure. A number of cancer cells, such as colorectal and pancreatic, have a prevalence of type 1 carbohydrate chains including sialyl-Le^a and di-sialyl-Le^a. Other cancer cells, such as breast, lung and ovarian, have a prevalence of type 2 carbohydrate chains including sialyl-Le^x.

Regarding in vitro aspects, as noted above, the present invention provides methods for inhibiting the binding of cancer cells to endothelia in a biological preparation. Representative examples of biological preparations include blood vessel and/or lymph vessel endothelia in combination with a malignancy. The endothelia and the malignancy may be in the form of tissue or cells removed from an organism, or cultured cells. In one embodiment, the method comprises incubating a biological preparation, which contains malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x and endothelial cells expressing a selectin, with an effective amount of at least one agent, such as an antibody, saccharide or glycoconjugate as described above. In another embodiment, the method comprises incubating malignant cells with at least one enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a

by the cells. Suitable enzyme inhibitors include inhibitors of glycosyltransferases. Representative examples of inhibitors for glycosyltransferases include inhibitors for fucosyltransferases (e.g., as described by Palcic et al., J. Biol. Chem. 264:17174-17181, 1989), for N-acetylglucosaminyltransferases (e.g., as described by Palcic et al., J. Biol. Chem. 265:6759-6769, 1990), and for sialyltransferases (e.g., as described by Broquet et al., J. Neurochem. 54:388-394, 1990; Karaivanova et al., Cancer Biochem. Biophys. 11:311-315, 1990).

In another embodiment, the method comprises incubating malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, with at least one enzyme that renders the carbohydrate on these cells unable to bind selectins. Suitable enzymes include glycosidases. Representative examples of glycosidases are sialidases (Rosen et al., Science 228:1005-1007, 1985) and fucosidases (Kobata, Methods in Enzymology 83:625-631, 1982). Enzymes possessing enhanced specificity and desirable characteristics can be detected using appropriate neoglycoproteins and anti-carbohydrate antibodies.

The present invention also provides use of the compositions described above in methods for inhibiting metastasis in a warm-blooded animal such as a human. In one embodiment, at least one agent, such as an antibody, saccharide or glycoconjugate as described above, is used to inhibit metastasis. In another embodiment, the composition comprises at least one enzyme inhibitor (as described above) that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by malignant cells. In another embodiment, the composition comprises an enzyme that alters sialyl-Le^a or di-sialyl-Le^a of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a such that malignant cells are incapable of binding to a selectin. It will be evident to those skilled in the art how to determine the optimal effective dose for a particular agent, enzyme inhibitor, or enzyme (e.g., based upon in vitro and in

vivo studies in non-human animals). A variety of routes of administration may be used. Typically, administration will be intravenous, intracavitory (e.g., in pleural or peritoneal cavities), or in the bed of a resected tumor.

An agent may be administered as a pharmaceutical composition, i.e., in combination with a pharmaceutically acceptable carrier or diluent, such as physiological saline. It will be recognized by those skilled in the art that an agent and a composition may be prepared in a sterile form. Moreover, an agent may be administered in combination with an immunotherapeutic or chemotherapeutic agent. When such a combination is desired, each substance may be administered sequentially, simultaneously, or combined and administered as a single composition. Diagnostic techniques, such as CAT scans for tumors, may be performed prior to and subsequent to administration to confirm effectiveness.

The present invention also provides compositions, and methods which use the same, comprising an agent capable of reacting with both sialyl-Le^a and sialyl-Le^x. Such agents include antibodies, microbial and mammalian carbohydrate binding proteins, such as receptors, adhesins, toxin subunits, and soluble selectins.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

GLYCOCONJUGATES AND ASSAYS

Synthetic glycoproteins (Neoglycoproteins)

Neoglycoproteins were produced by BioCarb AB (Lund, Sweden) by chemically coupling 10-20 moles of a specific oligosaccharide to 1 mole of nonglycosylated albumin, bovine (BSA) or human (HSA). The resulting synthetic glycoprotein (neoglycoprotein) contains multiple copies of the identical carbohydrate sequence, thereby producing a well characterized, mutivalent glycoconjugate which is extremely effective for studying carbohydrate-protein interactions. Depending on the size of the oligosaccharide, three different chemical spacer arms were used to couple the oligosaccharides to proteins 1) p-aminophenyl (PAP); 2) aminophenylethyl (APE); and 3) acetyl phenylene diamine were used to couple the shorter oligosaccharides to albumin since they will retain the anomeric configuration of the reducing sugars which may be involved in a potential binding site. APD was used to couple the larger sugars to protein by reductive amination, which converts the reducing sugar to an aminoalditol. These reduced sugars are designated by parenthesis in the APD conjugate presented in Table I.

TABLE I

Name	Structure
LNF I (H-type 2)	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)
LNF II (Le^a)	Galβ1-3GlcNAcβ1-4(Glc) 4 \| Fucα1

LNF III (Le^x)	Galβ1-4GlcNAcβ1-3Galβ1-4(Glc) 3 \| Fucα1
sLNFII (sLe^a)	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc) 4 \| Fucα1
sLNFIII (sLe^x)	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4(Glc) 3 \| Fucα1
LSTa	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Glc)
LSTc	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Glc)
3′ Sialyllactose	Neu5Acα2-3Galβ1-4(Glc)
6′ Sialyllactose	Neu5Acα2-6Galβ1-4(Glc)

Monoclonal Antibodies

The monoclonal antibodies employed include the following. HECA-452, a rat IgM [anti-CLA (Picker et al., J. Immunol. 145:3247-3255, 1990)] (Duijvestijn et al., Am. J. Path. 30:147-155, 1988); MECA-79, a rat IgM control [anti-peripheral lymph node addressin (Streeter et al., J. Cell Biol. 107:1853-1862, 1988)]; RB6-2C2, rat IgM control [Coffman and Weissman, J. Exp. Med. 153:269, 1981]; CL2 (anti-ELAM-1) (Picker et al., Nature 349:796-799, 1991), mouse IgG_1, kindly supplied by C. Wayne Smith (Houston, TX); Dreg-56, mouse IgG_1 [anti-human LECAM-1, (Kishimoto et al., Proc. Natl. Acad. Sci. USA 87:2244-2248, 1990)]; CSLEXI (TT-19, anti-sLNFIII) (Fukushima et al., Cancer Res. 44:5279-5286, 1984), a mouse IgM, kindly given by P. Terasaki (UCLA); and 1H10 (anti-sialyl-Le^a), a mouse IgG_1 developed by BioCarb.

Direct Binding of Antibodies to Synthetic Glycoproteins (Neoglycoproteins)

Synthetic glycoproteins were coated onto microtiter plates by filling each well with 100 ng of the neoglycoprotein in 100 μl of 0.15 M sodium chloride, 0.01

M sodium phosphate, 0.1% sodium azide, pH 7.4, (PBS-azide) overnight at 4°C. Standard enzyme-linked immunoassays (ELISA) were then performed on the solid phase carbohydrate structures using the appropriate antibody diluted to 10 μg/ml.

Production of ELAM-1 cDNA transfected cell lines

L1-2/pMRB107 cells (L1-2^{ELAM-1}) were prepared by transfecting the ELAM-1 gene into the murine pre-B cell line L1-2 (Gallatin et al., Nature 304:30-34, 1983). A cDNA clone encoding ELAM-1 was obtained from a cDNA library made from activated human umbilical vein endothelial cell cultures by polymerase chain reaction (PCR) amplification. The ELAM-1 gene was inserted downstream of the hCMV promoter in pMRB101 [a derivative of EE6 which contains the E. coli gpt gene (Mulligan and Berg, Proc. Natl.. Acad. Sci. USA 78:2072, 1981; Stephens and Corbett, N.A.R. 17:7110, 1989)]. DNA was introduced into L1-2 cells by electroporation and the cells selected for resistance to mycophenolic acid. A population of cells staining brightly for ELAM-1 were selected by FACS and cloned by limiting dilution. These cells are ELAM-1^{hi} LFA-1^{mod} $CD45^{hi}$ $CD44^{neg}$ LECAM-1^{neg}, differing from the parent cell line or control vector transfectants only in their expression of ELAM-1. L1-2/pMRB101 (L1-2^{vector}) cells are a similarly transformed derivative of L1-2 transfected with pMRB101 and lacking ELAM-1 expression.

Cell binding assays

One hundred microliter samples of each synthetic glycoconjugate in phosphate buffered saline (PBS), pH 7.2, were absorbed onto glass wells of 8-chamber slides (LabTek) for two hours at RT. For some experiments glass slides were pre-coated with rabbit anti-human serum albumin (Sigma) at 200μg/ml overnight at 4°C and washed with PBS prior to the addition of the glycoconjugate. After blocking with 5% NBS/ 10mM HEPES/Dulbecco's Modified

Eagles Medium (DMEM), pH 7.0 (CM), L1-2^{ELAM-1} or L1-2^{vector} cells were applied to each well (1.5 x 10^6/0.15 ml in CM). After a 25 minute incubation at RT on a rotating shaker at 50 rpm, the tops of the wells were removed and the slides washed 3x in DMEM and then fixed by incubation in 1.5% glutaraldehyde (Kodak)/DMEM. Three to six 100 x fields were counted for each data point.

Inhibition of Binding of ELAM-1 Containing Cells by Compounds

One hundred and twenty nanograms of sialyl-Le^a-HSA or sialyl-Le^x-HSA dissolved in 100 µl of phosphate-buffered saline were absorbed per well of an 8 chambered glass (LabTek) slide for 2 hours at room temperature. During this period, L1-2^{ELAM-1} cells were pre-incubated for 20 minutes on ice with increasing concentrations of sialyl-Le^a-HSA at 10^7 cells/ml. After washing and blocking the wells in Complete Medium (CM, 5% normal bovine serum, 10 mM HEPES, pH 7.0, DMEM), L1-2^{ELAM-1} cells pre-incubated with compounds were added (1 x 10^7 cells/ml) and incubated at room temperature while rotating at 50 rpm. After 25 minutes, slides were washed 3 times in Dulbecco's Modified Eagles Medium (DMEM) and then fixed in 1.5% glutaraldehyde/DMEM (Figure 5). The above experiments were repeated using a higher concentration of sialyl-Le^a-HSA to coat the wells (500 ng/100 µl) and different soluble multivalent compound (sialyl-Le^a-PA) for inhibition (Figure 6).

Inhibition of Intercellular Adhesion by Compounds

Normal human neutrophils or peripheral blood mononuclear cells (PBMC) (1-2 x 10^6/ml) are incubated in CM for 30 minutes at room temperature while rotating at 50 rpm on a layer of COS cells transfected with ELAM-1 cDNA. After washing, the binding of neutrophils is determined by directly counting the number of neutrophils bound per

transfected COS cell. For PBMC, non-adherent cells are removed by washing with DMEM and then adherent cells are removed by washing with a solution of 5 mM EDTA, 5 mM EGTA in PBS. Binding of monocytes is assessed by counting the number of adherent and non-adherent cells and determining the number of monocytes by their distinctive light-scatter profile with FACS analysis and the number of CLA^+ lymphocytes by staining with the anti-CLA mAb HECA-452 (Picker et al., Nature 349:796-799, 1991). Neutrophils and/or PBMC are pre-incubated with sialyl-Le^a-HSA or other compounds prior to incubation on the layer of ELAM-1 cDNA transfected COS cells. Inhibition of intercellular adhesion is determined as a percentage calculated by:

$$\frac{\text{number of bound cells in control - number of bound cells in test} \times 100}{\text{number of bound cells in control}}$$

Binding of Lymphocytes or LECAM-1 cDNA Transfectants to High Endothelial Venules

The interaction of the peripheral lymph node homing receptors (LECAM-1) with high endothelial venules is measured in a frozen section assay in which a suspension of lymphocytes and/or LECAM-1 transfected cell lines are incubated on frozen sections of lymphoid tissues for 20-30 minutes at 7°C (Stamper and Woodruff, J. Exp. Med. 144:828, 1976; Butcher et al., Eur. J. Immunol. 10:556, 1980) while rotating at 50 rpm. After glutaraldehyde fixation, the number of cells bound per HEV is determined microscopically. Sialyl-Le^a-HSA and other compounds are pre-incubated with lymphocytes or transfected cell lines, including LECAM-1 and ELAM-1 transfected L1-2 cells, prior to the assay, and the ability of the compounds to inhibit intercellular adhesion is determined as described above.

EXAMPLE 2

CARBOHYDRATE STRUCTURES RECOGNIZED BY ELAM-1

The sensitive binding assay described in Example 1 uses cells permanently transfected with ELAM-1 cDNA. The mouse pre-B cell line, L1-2, transfected with ELAM-1 cDNA (L1-2^{ELAM-1}), but not vector control cDNA, L1-2^{vector} expresses very high levels of ELAM-1. The ELAM-1 expressed by these cells is functional as L1-2^{ELAM-1} cells are adhesive for neutrophils and this adhesion is blocked by anti-ELAM-1 monoclonal antibodies. When added to glass slides coated with various synthetic glycoconjugates, L1-2^{ELAM-1} cells bound selectively to sialyl-Le^a and sialyl-Le^x neoglycoproteins, but not to a number of other glycoconjugates (see Table I for structures). L1-2^{ELAM-1} cells also bound, albeit more weakly, to Le^a neoglycoprotein. The binding to Le^a is significant as L1-2^{ELAM-1} cells bound poorly to Le^x and not at all to the glycoconjugates prepared with the structural analogs such as LNF I. That L1-2^{ELAM-1} cells did not bind other monosialylated carbohydrates, such as 3'SL, 6'SL, LSTa or LSTc demonstrates that the binding to sialyl-Le^a and sialyl-Le^x is not due to non-specific charge effects, but rather reflects specific structural features of these oligosaccharides. The low level of binding of ELAM-1 transfectants to Le^a is consistent with an essential role of fucose in recognition, but shows that neuraminic acid (also known as sialic acid) also plays a key role.

Hard Sphere Exo-Anomeric (HSEA) Calculations

Conformational models of the oligosaccharides in solution were obtained by HSEA calculations. Hydroxyl groups are represented by the oxygen atoms. A fixed bond angle of 117° was used for the glycosidic linkages. The energy calculated by a HSEA potential (Bock, Pure Appl. Chem. 55:605-622, 1983), was minimized using simultaneous variation of dihedral angles (multi-dimensional binary

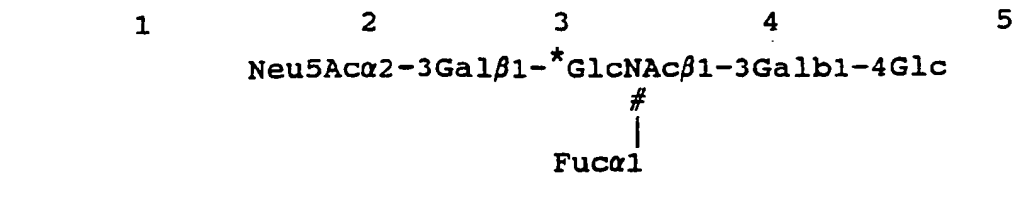
chop). This algorithm shows a slow convergence near a local minimum when compared to other methods utilizing the first and second derivative, but has the advantage of allowing a large initial search area of the conformational space, whereby the chances of finding the lowest local minima increases. Other applications of this program are described in Kumlien et al. (Arch. Biochem. Biophys. 269:678-689, 1989) and Wreslander et al. (Glycoconjugate J. 7:85-100, 1990).

Carbohydrate Structures that Inhibit the Binding of ELAM-1 Dependent Intercellular Adhesion

Sialyl-Le^a-HSA in solution blocks 10% of binding of ELAM-1 transfected cells (L1-2^{ELAM-1}) to either immobilized sialyl-Le^x-HSA or immobilized sialyl-Le^a-HSA. As binding to either carbohydrate structure is blocked by sialyl-Le^a-HSA, only one carbohydrate-binding site exists in ELAM-1 which recognizes a carbohydrate domain common to both sialyl-Le^a and sialyl-Le^x.

Graphic Representation of the Carbohydrate Epitope for ELAM-1

The dihedral angles for sialyl-Le^a and sialyl-Le^x hexassacharide determined by the HSEA calculations are presented in Table II. It should be noted that these are theoretical approximations of the native conformation and the disclosure is not restricted to these bond angles. The dihedral angles are specified by the designation of the four atoms defining it. These 4-character designations are made up of the chemical symbol, 2 characters for the number in the monosaccharide (and possible extra specification, e.g., to distinguish atoms of the same type bonded to the same carbon), and the number of the monosaccharide residue in the oligosaccharide. The last number is defined below:



*=3 in case of sialyl-Le^a; 4 in case of sialyl-Le^x.
#=4 in case of sialyl-Le^a; 3 in case of sialyl-Le^x.

TABLE II

Dihedral Angle				Sialyl-Le^a(°)	Sialyl-Le^x(°)
C1 1 - C2	1 - 02	1 - C3	2	188.6240387	189.0001221
C2 1 - 02	1 - C3	2 - H3	2	350.8763428	349.9999695
H1 2 - C1	2 - 01	2 - C*	3	51.3739777	53.2507896
C1 2 - 01	2 - C*	3 - H*	3	15.3727636	8.8746433
H1 3 - C1	3 - 01	3 - C3	4	57.8722878	57.8755035
C1 3 - 01	3 - C3	4 - H3	4	350.6306458	350.6243591
H1 4 - C1	4 - 01	4 - C4	5	55.3822517	55.4999657
C1 4 - 01	4 - C4	5 - H4	5	2.6262217	1.6283444
H1 6 - C1	6 - 01	6 - C#	3	49.7558937	49.8749161
C1 6 - 01	6 - C#	3 - H#	3	18.8635368	23.9994240
09 1 - C9	1 - C8	1 - C7	1	178.1011200	178.2247772
08 1 - C8	1 - C7	1 - C6	1	299.6979065	300.0729675
07 1 - C7	1 - C6	1 - H6	1	180.4196625	182.0449371
06 2 - C6	2 - C5	2 - H5	2	303.0703125	300.8233032
06 3 - C6	3 - C5	3 - H5	3	289.2540588	294.3686523
06 4 - C6	4 - C5	4 - H5	4	306.4527283	306.4491882
06 5 - C6	5 - C5	5 - H5	5	296.1771851	178.0553131
H6A6 - C6	6 - C5	6 - H5	6	179.6257324	179.8791046

* = 3 in case of sialyl-Le^a; 4 in case of sialyl-Le^x.
# = 4 in case of sialyl-Le^a; 3 in case of sialyl-Le^x.

EMIN (sialyl-Le^a) = -15.7628746 kcal/mol
EMIN (sialyl-Le^x) = -14.9528790 kcal/mol

For control purposes the full program output is presented, while the relevant accuracy for comparison with experimental data cannot be expected to be higher than ± for the angles. The error in the HSEA energy value can be expected to lie in the first decimal, when given in kcal/mol. These energy values are of interest when comparing different potential functions, etc., but does not lend itself easily to comparison with energies

determined from experiments. A large negative value does, however, show that the attractive van der Waals forces dominate the calculations, giving support for the use of the HSEA approximation (positive energies indicate strong steric forces that may distort bond lengths and angles, which are assumed constant in HSEA). The resulting structures are represented graphically in Figure 1.

The calculations show high similarity in the corresponding dihedral angles for the two structures, also at the bonds with different linkage between the N-acetyl-glucosamine and the fucose and sialic acid residues, respectively. The different dihedral angles for the hydroxymethyl group in the 6-position of the glucose residue at the reducing terminal (296.2° and 178.1°) is a result of the very nearly equal energies for this molecular group after a rotation of 120°. As this group is far away from the linkages differing between sialyl-Le^a and sialyl-Le^x, its direction is of no importance for the conformational structure in this region. Computer-generated images of the structures are represented graphically in Figure 1. The conformations indicate that the structures show a high degree of similarity in both the non-reducing and reducing terminal parts, respectively. In particular, the structures of the terminal carbohydrate sequence up to but not including the N-acetyl group on the internal GlcNAc residue, show a high degree of homology and may represent the domain recognized by both ELAM-1 and the monoclonal antibody HECA-452. This area of structural homology is particularly useful for the design of potential anti-inflammatory drugs.

Examples of other carbohydrate-binding proteins that recognize type 1 and type 2 chain isomers are the antibodies $E_1$23-48 and $E_1$66-18 which bind the blood group B antigen (Hansson et al., J. Biol. Chem. 258:4091, 97, 1983) and the lectin, Griffonia simplicifolia IV, which recognizes both Le^b and Le^y antigens (Spohr et al., Can. J. Chem. 63:2644-52, 1985).

The recognition of the sialyl-Le^a antigen and sialyl-Le^x antigen, by ELAM-1, may be of pathologic importance. Mucins containing these structures are elevated in the sera of cancer patients, including gastrointestinal, pancreatic, and breast cancer patients (Magnani et al., J. Biol. Chem. 257:14365-369, 1982; Magnani et al., Cancer Res. 43:5481-92, 1983). Preliminary experiments indicate that some sialyl-Le^a- and sialyl-Le^x-containing mucins are recognized by ELAM-1 transfectants. By interacting with ELAM-1 on venules in acute and chronically inflamed tissues and interfering with the recruitment of leukocytes to these locations, these mucins secreted by tumors may contribute to the immunodepressed state of cancer patients.

EXAMPLE 3

CARBOHYDRATE STRUCTURES RECOGNIZED BY LECAM-1

Production of LECAM-1 cDNA transfected cells

A human LECAM-1 cDNA transfected cell line (L1-$2^{LECAM-1}$) was prepared by transfecting the LECAM-1 gene into the murine pre-B cell line L1-2 (Gallatin et al., Nature 304:30-34, 1983). A cDNA clone encoding LECAM-1 was obtained from a cDNA library made from peripheral blood lymphocytes by polymerase chain reaction amplification. The LECAM-1 gene was inserted downstream of the hCMV promoter in pMRB101 [a derivative of EE6 which contains the E. coli gpt gene (Mulligan and Berg, Proc. Natl. Aca. Sci. USA 78:2072-2076, 1981; Stephens and Cockett, N.A.R. 7:7110, 1989)]. DNA was introduced into L1-2 cells by electroporation and the cells selected for resistance to mycophenolic acid. A population of cells staining brightly for LECAM-1 were selected by FACS and cloned by limiting dilution. These cells are LECAM-1^{hi} LFA-1^{mod} $CD45^{hi}$ $CD44^{neg}$, differing from the parent cell line or control vector transfectants only in their expression of LECAM-1. L1-2/PMRB101 (L1-2^{vector}) cells

are a similarly transformed derivative of L1-2 transfected with pMRB101 and lack LECAM-1 expression.

Cell binding assay

One hundred microliter samples of each neoglycoconjugate in phosphate buffered saline (PBS), pH 7.2, were absorbed onto glass wells of 8-chamber slides (LabTek) for two hours at room temperature. After blocking with 5% NBS, 10 mM HEPES, Dulbecco's Modified Eagles Medium (DMEM), pH 7.0 (CM), L1-2$^{LECAM-1}$, L1-2^{vector} or L1-2^{ELAM-1} cells were applied to each well (1.5×10^6 cells in 0.15 ml CM). Mouse lymphocytes isolated from mesenteric lymph nodes were also tested at 3×10^6 cells in 0.15 ml. In some cases, cells were pre-incubated with monoclonal antibody MEL-14 (Gallatin et al., 1983, supra) at 150 μg/ml/10^7 cells and washed prior to testing. After a 25-minute incubation at room temperature on a rotating shaker at 50 rpm, the tops of the wells were removed and the slides washed 3x in DMEM and then fixed by incubation in 1.5% glutaraldehyde (Kodak) in DMEM. Three to six 100 x fields were counted for each data point and the average and standard error are reported. Data reported are from representative experiments which were performed 2-5 times with similar results.

Lymphocytes bind to Sialyl-Le^x and Sialyl-Le^a containing neoglycoconjugates via LECAM-1

The determination that LECAM-1 cross-reacts with these ELAM-1 ligands (described in Example 1) was performed by repeating this adherence assay with L1-2 cells transfected with human LECAM-1 cDNA (L1-2$^{LECAM-1}$) as well as with normal mouse lymphocytes which express high levels of mouse LECAM-1. Mouse lymphocytes bound sialyl-Le^x and sialyl-Le^a, but not LNF I, Le^a, Le^x or neoglycoconjugates containing 3' sialyllactose, 6' sialyllactose, or LSTa. Binding of mouse lymphocytes was blocked by anti-mouse LECAM-1 MAB MEL-14 (Gallatin et al.,

1983, supra) demonstrating that the adhesion observed is via LECAM-1 (Figure 8). L1-2$^{LECAM-1}$ cells all bind slightly better to sialyl-Le^a than sialyl-Le^x containing conjugates over a wide range of cell concentrations; both ELAM-1 and LECAM-1 appear to display similar relative binding abilities to these two carbohydrate ligands (Figure 7).

It is evident from the above results, that compositions can be employed which can be used to modulate the homing of leukocytes, particularly lymphocytes, to sites of inflammation. These compounds can be readily prepared by conventional ways and can be effective for the treatment of a variety of diseases, both prophylactically and therapeutically.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modification may be made without deviating from the spirit and scope of the invention.

Claims

1. A method for modulating the binding of leukocytes or platelets to endothelial cells, said method comprising:

adding to a combination of cells comprising leukocytes and endothelial cells expressing selectins or carbohydrate ligands thereof, in an amount sufficient to modulate the binding of leukocytes to endothelial cells, a compound capable of being cross-reactive and/or competitive with sialyl-Le^x, sialyl-Le^a or the cutaneous lymphocyte-associated antigen in binding to a selectin, wherein said compound is other than sialyl-Le^x when said selectin is ELAM-1.

2. A method according to claim 1 wherein said compound comprises sialic acid and fucopyranose bonded to a group comprising a conformationally constrained chain of at least 2 atoms.

3. A method according to claim 2 wherein said group comprises at least one of galactose, glucose, or derivative thereof.

4. A method according to claim 3 wherein said galactose is bonded as the β-anomer to a glucose, glucosamine or N-acetyl glucosamine.

5. A method according to claim 4 wherein said compound is sialyl-Le^a or derivative thereof.

6. A compound other than sialyl-Le^x comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to ELAM-1, LECAM-1 or GMP-140 for use within a method for

inhibiting the infiltration of leukocytes into an inflammation site of a host.

7. A compound according to claim 6 wherein said compound is the polysaccharide of the cutaneous lymphocyte-associated antigen.

8. A compound comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin for use within a method for inhibiting the infiltration of lymphocytes into an inflammation site of a host.

9. A compound comprising Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin for use within a method for inhibiting the binding of platelets to endothelial cells.

10. A compound other than a naturally occurring sialyl-Le^a or sialyl-Le^x antigen comprising:

sialic acid and fucopyranose bonded to a group comprising a conformationally constrained chain.

11. A compound according to claim 10 wherein said group comprises at least one of galactose, glucose or derivatives thereof and said sialic acid and fucopyranose are separated by a chain of at least 2 carbon atoms.

12. A compound other than a naturally occurring sialyl-Le^a or sialyl-Le^x antigen comprising:

Neu5Acα2-3Galβ1-x[Fucα1-y]GlcNAc, wherein one of x and y is 3 and the other 4, or derivative thereof capable of binding to a selectin.

13. A compound according to claim 11 bonded to a carrier molecule through a spacer group.

14. A compound according to claim 13 wherein said carrier molecule is a polymer.

15. A compound other than a sialyl-Le^x or sialyl-Le^a antigen comprising:

Neu5Acα2-3Galβ1-x[Fucα1-y]R, wherein one of x and y is 3 and the other 4, R is a saccharide or derivative, said compound being capable of binding to a selectin.

16. A compound according to claim 15 wherein said R is glucose or a derivative.

17. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin, comprising:

incubating the biological preparation with at least one agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin, wherein said agent is other than sialyl-Le^x when said malignant cells express sialyl-Le^x.

18. The method of claim 17 wherein the agent is a saccharide, a glycoconjugate or an antibody that inhibits the binding of sialyl-Le^a, di-sialyl-Le^a, or sialyl-Le^x, to a selectin.

19. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1, comprising:

incubating the biological preparation with at least one agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1, wherein said agent is

other than sialyl-Le^x when said malignant cells express sialyl-Le^x.

20. The method of claim 19 wherein the agent is a saccharide, a glycoconjugate or an antibody that inhibits the binding of sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x to ELAM-1.

21. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to endothelial cells expressing a selectin, comprising:

incubating said malignant cells with at least one enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by said malignant cells.

22. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to endothelial cells expressing ELAM-1, comprising:

incubating said malignant cells with at least one enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by said malignant cells.

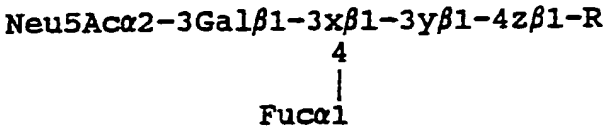
23. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to endothelial cells expressing a selectin, comprising:

incubating said malignant cells with at least one enzyme that alters sialyl-Le^a or di-sialyl-Le^x of said malignant cells such that said malignant cells are incapable of binding to a selectin.

24. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to endothelial cells expressing ELAM-1, comprising:

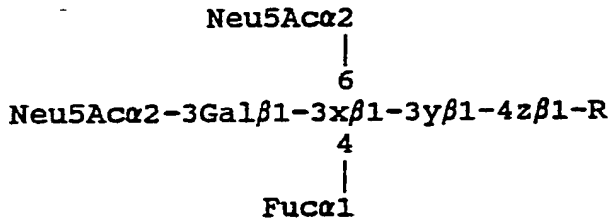
incubating said malignant cells with at least one enzyme that alters sialyl-Le^a or di-sialyl-Le^x of said malignant cells such that said malignant cells are incapable of binding to ELAM-1.

25. A compound having the formula:



wherein x, y and z are independently selected from saccharides or y or z or both are not present, and R is H, OH, lipid, ceramide, or one or more amino acids, with the proviso that x, y and z are not present in the combination wherein x is GlcNAc, y is Gal and z is Glc.

26. A compound having the formula:



wherein x, y and z are independently selected from saccharides or y or z or both are not present, and R is H, OH, lipid, ceramide, or one or more amino acids, with the proviso that x, y and z are not present in the combination wherein x is GlcNAc, y is Gal and z is Glc.

27. An agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin, wherein said agent is other than sialyl-Le^x when said malignant cells express sialyl-Le^x, for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites.

28. The agent of claim 27 wherein said agent is a saccharide, a glycoconjugate or an antibody that inhibits the binding of sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x to a selectin.

29. An agent that inhibits the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1, wherein said agent is other than sialyl-Le^x when said malignant cells express sialyl-Le^x, for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites by hematogeneous metastases.

30. The agent of claim 29 wherein said agent is a saccharide, a glycoconjugate or an antibody that inhibits the binding of sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x to ELAM-1.

31. An enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to secondary sites.

32. An enzyme inhibitor that inhibits the biosynthesis of sialyl-Le^a or di-sialyl-Le^a by malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to secondary sites by hematogeneous metastases.

33. An enzyme that alters sialyl-Le^a or di-sialyl-Le^a of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a such that said malignant cells are incapable of binding to a selectin, for use within a method for inhibiting in a warm-

blooded animal the spread of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to secondary sites.

34. An enzyme that alters sialyl-Le^a or di-sialyl-Le^a of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a such that said malignant cells are incapable of binding to a selectin, for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a or di-sialyl-Le^a, to secondary sites by hematogeneous metastases.

35. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing a selectin, comprising:

incubating the biological preparation with at least one agent capable of reacting with both sialyl-Le^a and sialyl-Le^x.

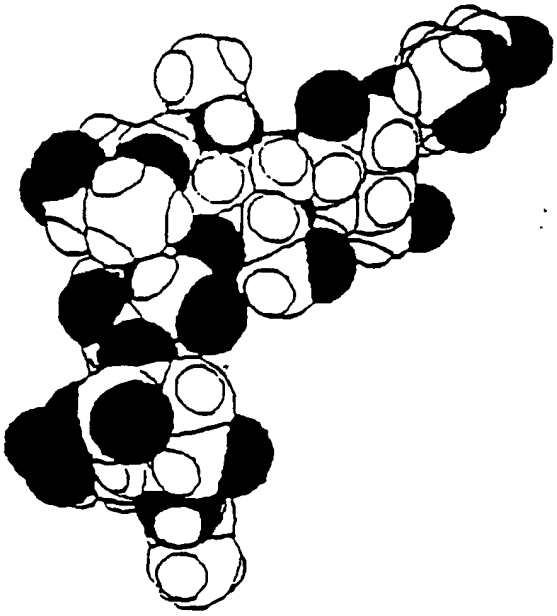
36. A method for inhibiting within a biological preparation the binding of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to endothelial cells expressing ELAM-1, comprising:

incubating the biological preparation with at least one agent capable of reacting with both sialyl-Le^a and sialyl-Le^x.

37. An agent capable of reacting with both sialyl-Le^a and sialyl-Le^x for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites.

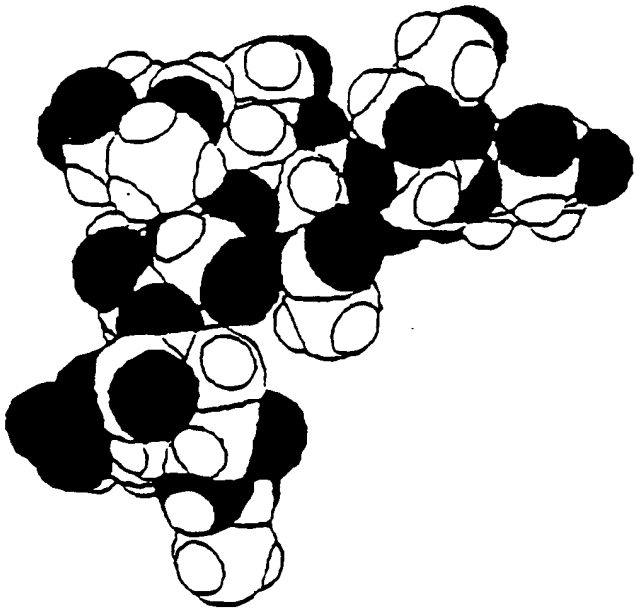
38. An agent capable of reacting with both sialyl-Le^a and sialyl-Le^x for use within a method for inhibiting in a warm-blooded animal the spread of malignant cells expressing sialyl-Le^a, di-sialyl-Le^a or sialyl-Le^x, to secondary sites by hematogeneous metastases.

39. A compound according to any one of claims 10-16 and 25-26 for use in the manufacture of a medicament.



Sialyl Le^x Hexasaccharide

FIG. 1A



Sialyl Le^a Hexasaccharide

FIG. 1B

Figure 2

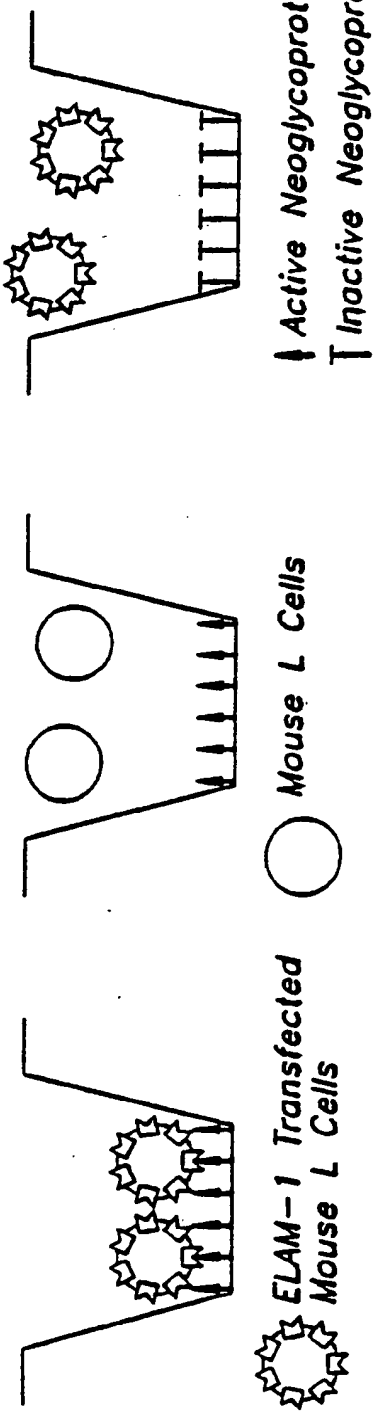


Figure 3

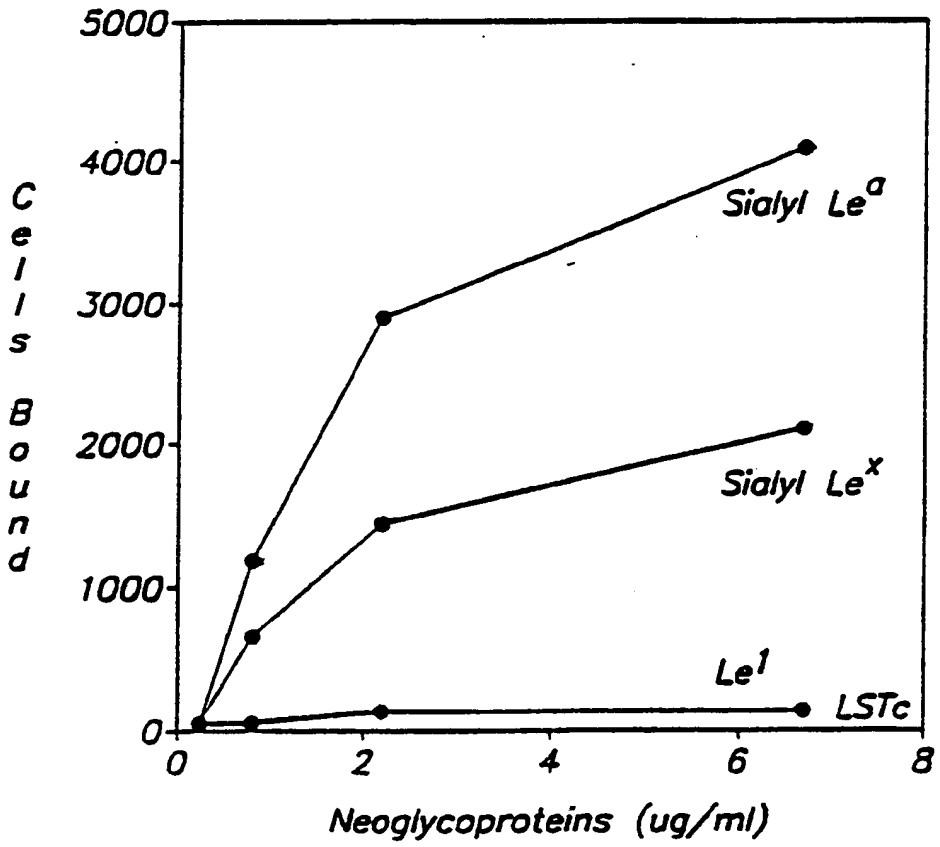
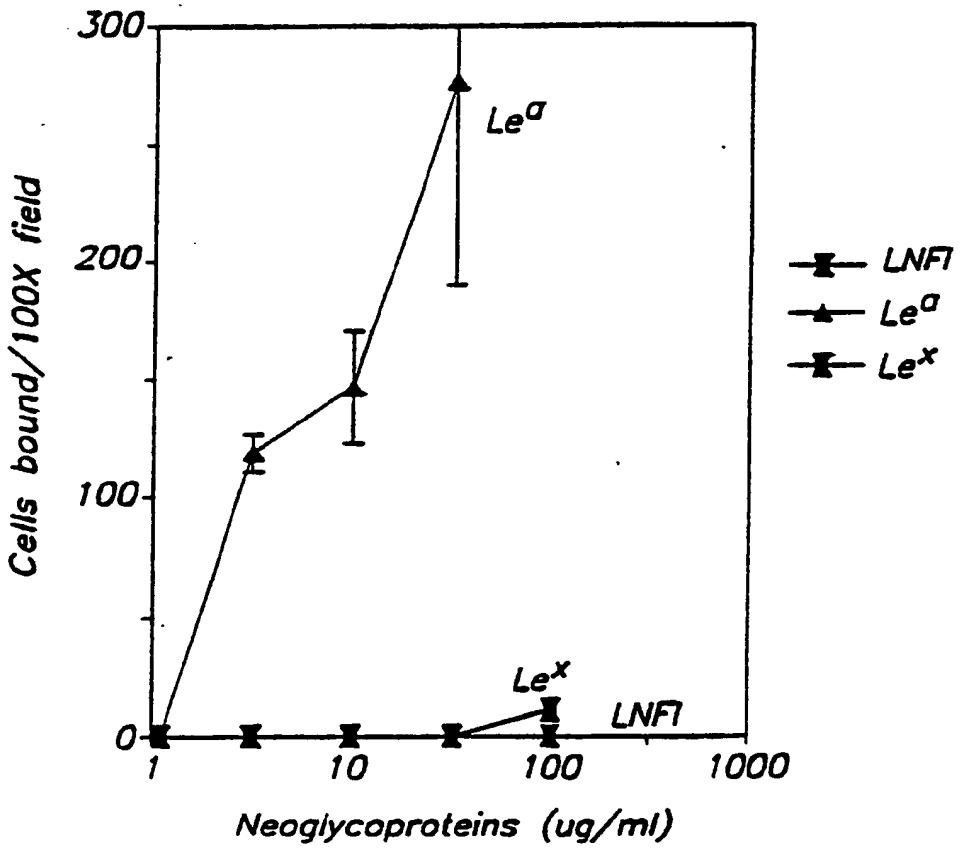

5000
4000
3000
2000
1000
0
Cells Bound
Sialyl Le^a
Sialyl Le^x
Le^1
LSTc
0
2
4
6
8
Neoglycoproteins (ug/ml)

Figure 4



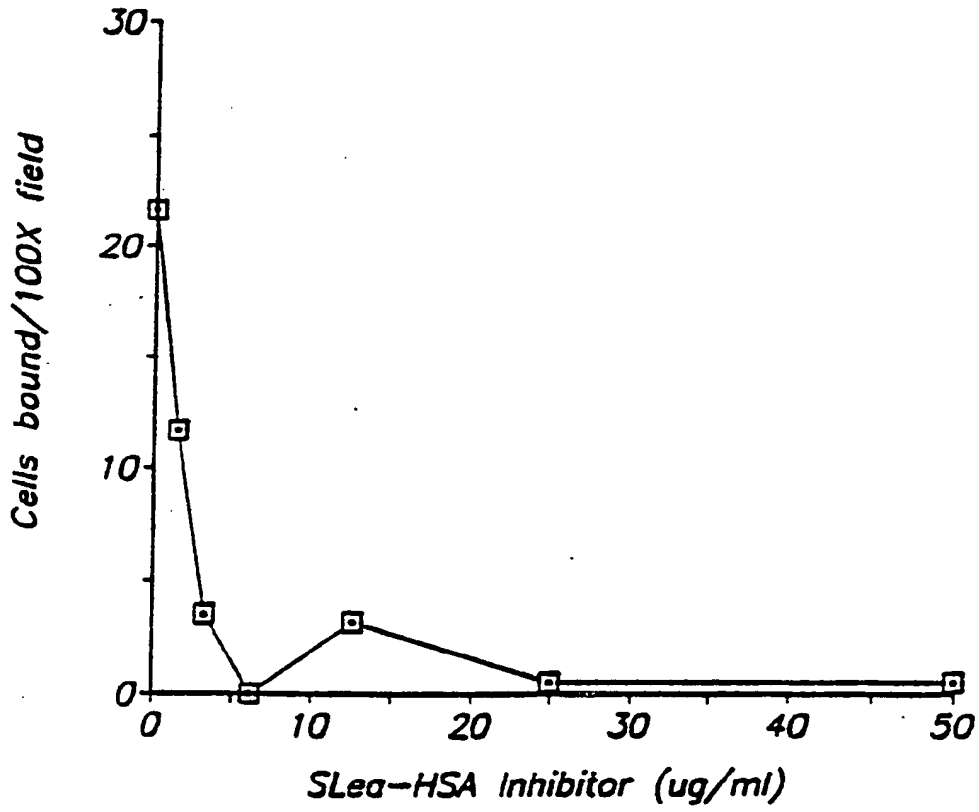

30
20
10
0
Cells bound/100X field
0
10
20
30
40
50
SLea-HSA Inhibitor (ug/ml)

Figure 5

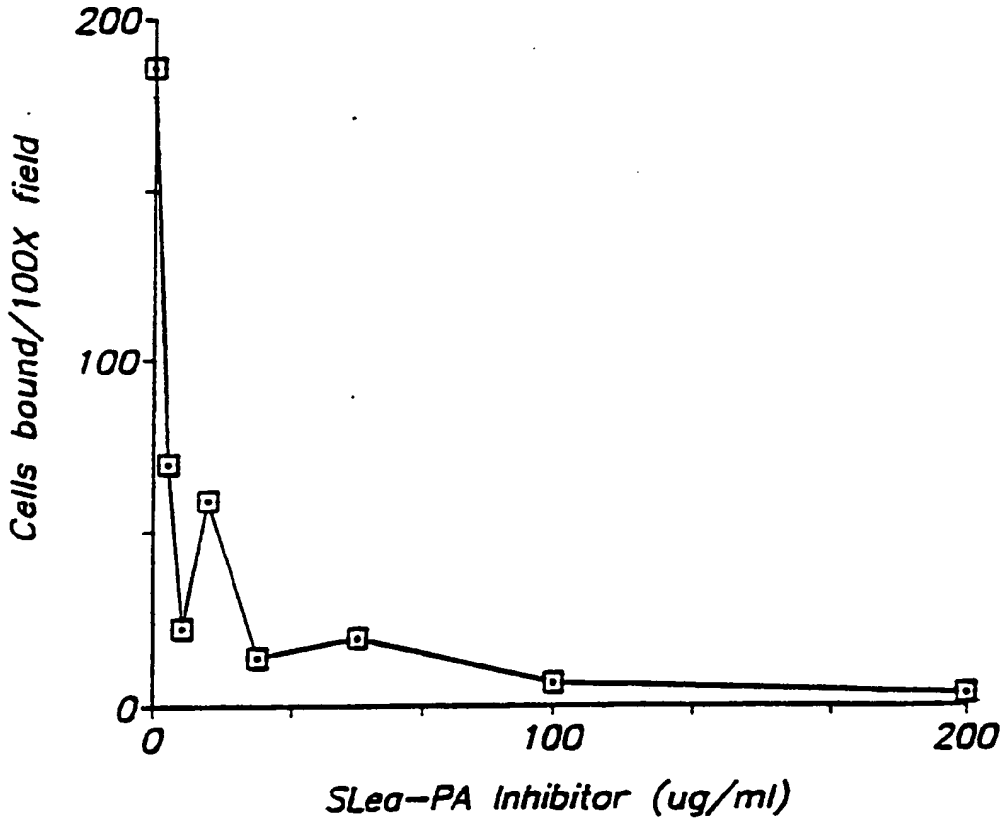


Figure 6

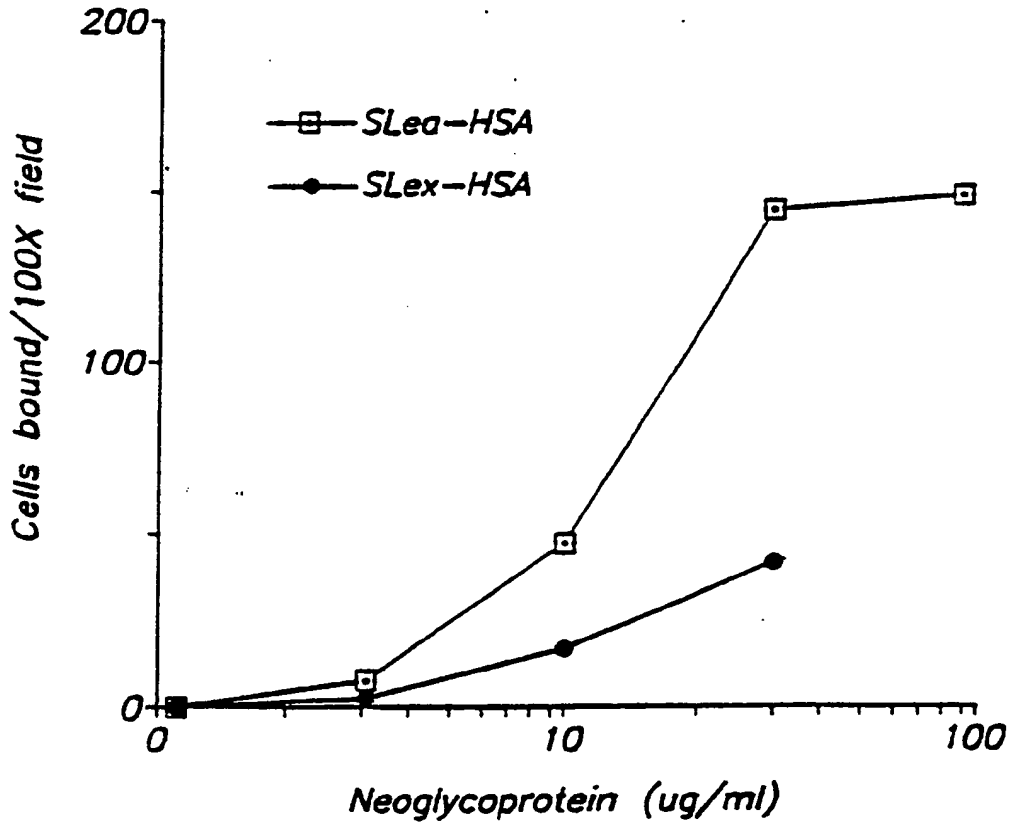

200
100
0
Cells bound/100X field
SLea-HSA
SLex-HSA
0
10
100
Neoglycoprotein (ug/ml)

Figure 7

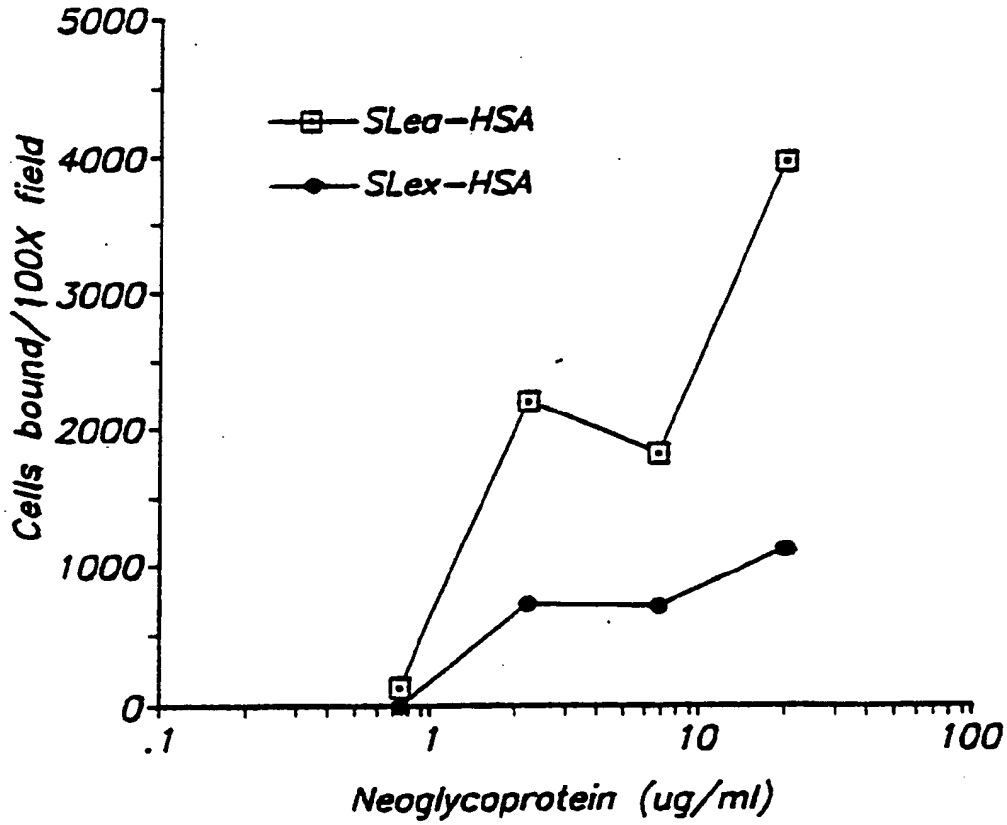


Figure 8

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(57) Abstract

Novel methods and compositions are provided for modulating homing of leukocytes, particularly lymphocytes, where the compounds are cross-reactive with or contain Neu5Ac2-3Galβ1-x[Fucα1-y]GlcNAc, where one of x and y is 3 and the other is 4. These compounds may be administered to a host associated with inflammation, to avoid the deleterious effects of leukocyte infiltration and for directing molecules to such sites. In addition, methods and compositions are disclosed for the inhibition of cancer metastases mediated by endothelial adhesion molecules. The present invention discloses that sialyl-Le^a and di-sialyl-Le^a, which are expressed at the surface of cancer cells, function as a binding partner for selectins, such as ELAM-1, which are expressed at the surface of endothelial cells. The present invention also discloses that selectins, such as ELAM-1, LECAM-1 and GMP-140, bind a carbohydrate domain common to both sialyl-Le^a and sialyl-Le^x. Antibodies, saccharides, glycoconjugates, enzymes, enzyme inhibitors and other molecules may be used in the methods of the present invention to inhibit the binding of malignant cells to endothelial cells for a variety of purposes *in vivo* and *in vitro*.

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Int.Cl.5 C 07 K 15/14 C 07 H 13/04 A 61 K 31/70
A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 07 H A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	CELL vol. 63, 1990, pages 861 - 863 B.K. BRANDLEY ET AL 'Carbohydrate Ligands of LEC Cell Adhesion Molecules' see whole document ---	1-20,25 -30,39
Y	SCIENCE vol. 250, 1990, pages 1130 - 1132 M.L. PHILLIPS ET AL 'ELAM-1 Mediates Cell Adhesion by Recognition of a Carbohydrate Ligand Sialyl-Le-x' see whole document ---	1-20,25 -30,39
Y	SCIENCE vol. 250, 1990, pages 1132 - 1135 G. WALZ ET AL 'Recognition by ELAM-1 of Sialyl-Le-x Determinant on Myeloid and Tumor Cells' see whole document --- -/-	1-20,25 -30,39

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02-11-1992	03. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	BRENNAN J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CELL vol. 63, 1990, pages 467 - 474 E. LARSEN ET AL 'PADGEM-Dependent Adhesion of Platelets to Monocytes and Neutrophils is Mediated by a Lineage-Specific Carbohydrate, LNF III (CD15)' see page 469, column 2 - page 470, column 2 ---	1-20,25 -30,39
Y	CELL vol. 63, 1990, pages 475 - 484 J.B. LOWE ET AL 'ELAM-1-Dependent Cell Adhesion to Vascular Endothelium Determined by a transfected Human Fucosyltransferase cDNA' see whole document ---	1-20,25 -30,39
Y	WO,A,9013300 (BIOGEN) 15 November 1990 see page 29, line 10 - line 17; claim 63 ---	1-20,25 -30,39
X	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY vol. 112, 1990, pages 3693 - 3695 K.C. NICOLAOU ET AL 'Total Synthesis of the Tumor-Associated Le-x Family of Glycosphingolipids' see the whole document ---	6-16
X	JOURNAL OF CANCER vol. 45, 1990, pages 1022 - 1027 C.K. CHING ET AL 'Purification and Characterisation of a Peanut-Agglutin-Binding Pancreatic-Cancer-Related Serum Mucus Glycoprotein' see p.1026, Fig.8 ---	6-16,25
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 172, 1990, pages 1349 - 1356 L. CORRAL ET AL 'Requirements for Sialic Acids on Neutrophils in a GMP-140 (PADGEM) Mediated Adhesive Interaction with Activated Platelets' see p.1349, Summary ---	1
A	EP,A,0408859 (OTSUKA) 23 January 1991 see claims 45-58 ---	1
P,X	WO,A,9119502 (CYTEL) 26 December 1991 see the whole document ---	1-20,25 -30,39
P,X	WO,A,9201718 (REGENTS OF THE BOARD OF THE UNIVERSITY OF OKLAHOMA) 6 February 1992 see page 32, line 19 - page 37; claims 9-18 and 29-38 --- -/-	1-20,25 -30,39

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES(USA) vol. 88, November 1991, pages 10372 - 10376 D. TYRRELL ET AL 'Structural Requirements for the Carbohydrate Ligand of E-Selectin' see the whole document ---	1-20,25 -30,39
E	WO,A,9207572 (REGENTS OF THE UNIVERSITY OF MICHIGAN) 14 May 1992 see page 12, line 2 - page 13, line 25; claims 1-3 -----	1-16,39

INTERNATIONAL SEARCH REPORT

:rnational application No.
PCT/US92/03192

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims: 1-20, 25-30, 39.
2. Claims: 21-22, 31-32.
3. Claims: 23-24, 33,34.
4. Claims: 35-38.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1. Claims: 1-20, 25-30, 39.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9203192 SA 60190

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/02/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 9013300	15-11-90	AU-A-	6049290	29-11-90
		CA-A-	2031518	29-10-90
		CA-A-	2063244	28-10-91
		EP-A-	0458911	04-12-91
		JP-T-	4502859	28-05-92
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		AU-A-	8102991	07-01-92
		WO-A-	9119501	26-12-91
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